

EXAMPLE 21

The Fate Of The Target RNA In The Invader-Directed Cleavage Reaction

5 In this example, the fate of the RNA target in the invader-directed cleavage reaction was examined. As shown above in Example 1D, when RNAs are hybridized to DNA oligonucleotides, the 5' nucleases associated with DNA polymerases can be used to cleave the RNAs; such cleavage can be suppressed when the 5' arm is long or when it is highly structured [Lyamichev *et al.* (1993) *Science* 260:778 and U.S. Patent No. 5,422,253, the disclosure of which is herein incorporated by reference]. In this experiment, the extent to which the RNA target would be cleaved by the cleavage agents when hybridized to the detection oligonucleotides (*i.e.*, the probe and invader oligonucleotides) was examined using reactions similar to those described in Example 20, performed using fluorescein-labeled RNA as a target.

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15 Transcription reactions were performed as described in Example 20 with the exception that 2% of the UTP in the reaction was replaced with fluorescein-12-UTP (Boehringer Mannheim) and 5.3 µg of the amplicon was used in a 100 µl reaction. The transcription reaction was incubated for 2.5 hours, after which the DNA template was destroyed by the addition of 5-6 µl of RQ1 RNase-free DNase (1 unit/µl) according to the RiboMAX™ kit instructions. The organic extraction was omitted and the RNA was collected by the addition of 10 µl of 3M NaOAc, pH 5.2 and 110 µl of 100% isopropanol. Following a 5 min. incubation at 4°C, the precipitate was collected by centrifugation, washed once with 80% ethanol and dried under vacuum. The resulting RNA was dissolved in 100 µl of nuclease-free water. 50% of the sample was purified by electrophoresis through a 8% denaturing polyacrylamide gel (19:1 cross-linked), containing 7 M urea, in a buffer of 45 mM Tris-Borate, pH 8.3, 1.4 mM EDTA. The gel slice containing the full-length material was excised and the RNA was eluted by soaking the slice overnight at 4°C in 200 µl of 10 mM Tris-Cl, pH 8.0, 0.1 mM EDTA and 0.3 M NaOAc. The RNA was then precipitated by the addition of 2.5 volumes of 100% ethanol. After incubation at -20°C for 30 min., the precipitates

were recovered by centrifugation, washed once with 80% ethanol and dried under vacuum. The RNA was dissolved in 25 µl of nuclease-free water and then quantitated by UV absorbance at 260 nm.

Samples of the purified RNA target were incubated for 5 or 30 min. in reactions that duplicated the Cleavase® A/G and DNAPTth invader reactions described in Example 20 with the exception that the reactions lacked probe and invader oligonucleotides. Subsequent analysis of the products showed that the RNA was very stable, with a very slight background of non-specific degradation, appearing as a gray background in the gel lane. The background was not dependent on the presence of enzyme in the reaction.

Invader detection reactions using the purified RNA target were performed using the probe/invader pair described in Example 20 (SEQ ID NOS:57 and 58). Each reaction included 500 fmole of the target RNA, 5 pmoles of the fluorescein-labeled probe and 10 pmoles of the invader oligonucleotide in a buffer of 10 mM MOPS, pH 7.5 with 150 mM LiCl, 4 mM MnCl₂, 0.05% each Tween-20 and Nonidet-P40 and 39 units RNAsin® (Promega). These components were combined and warmed to 50°C and the reactions were started by the addition of either 53 ng of Cleavase® A/G or 5 polymerase units of DNAPTth. The final reaction volume was 10 µl. After 5 min at 50°C, 5 µl aliquots of each reaction were removed to tubes containing 4 µl of 95% formamide, 10 mM EDTA and 0.02% methyl violet. The remaining aliquot received a drop of ChillOut® evaporation barrier and was incubated for an additional 25 min. These reactions were then stopped by the addition of 4 µl of the above formamide solution. The products of these reactions were resolved by electrophoresis through separate 20% denaturing polyacrylamide gels (19:1 cross-linked), containing 7 M urea, in a buffer of 45 mM Tris-Borate, pH 8.3, 1.4 mM EDTA. Following electrophoresis, the labeled reaction products were visualized using the FMBIO-100 Image Analyzer (Hitachi), with the resulting imager scans shown in Figures 53A (5 min reactions) and 53B (30 min. reactions).

In Figure 53 the target RNA is seen very near the top of each lane, while the labeled probe and its cleavage products are seen just below the middle of each panel.

5 The FMBIO-100 Image Analyzer was used to quantitate the fluorescence signal in the probe bands. In each panel, lane 1 contains products from reactions performed in the absence of a cleavage agent, lane 2 contains products from reactions performed using Cleavase® A/G and lane 3 contains products from reactions performed using DNAPTth.

10 Quantitation of the fluorescence signal in the probe bands revealed that after a 5 min. incubation, 12% or 300 fmole of the probe was cleaved by the Cleavase® A/G and 29% or 700 fmole was cleaved by the DNAPTth. After a 30 min. incubation, Cleavase® A/G had cleaved 32% of the probe molecules and DNAPTth had cleaved 70% of the probe molecules. (The images shown in Figures 53A and 53B were printed with the intensity adjusted to show the small amount of background from the RNA degradation, so the bands containing strong signals are saturated and therefore these images do not accurately reflect the differences in measured fluorescence)

15 The data shown in Figure 53 clearly shows that, under invasive cleavage conditions, RNA molecules are sufficiently stable to be detected as a target and that each RNA molecule can support many rounds of probe cleavage.

EXAMPLE 22

Titration Of Target RNA In The Invader-Directed Cleavage Assay

20 One of the primary benefits of the invader-directed cleavage assay as a means for detection of the presence of specific target nucleic acids is the correlation between the amount of cleavage product generated in a set amount of time and the quantity of the nucleic acid of interest present in the reaction. The benefits of quantitative detection of RNA sequences was discussed in Example 20. In this example, we
25 demonstrate the quantitative nature of the detection assay through the use of various amounts of target starting material. In addition to demonstrating the correlation between the amounts of input target and output cleavage product, these data graphically show the degree to which the RNA target can be recycled in this assay